AD_____

Award Number: DAMD17-01-1-0282

TITLE: Dissecting the Mechanisms of T Cell Tolerance for More Effective Breast Cancer Vaccine Development

PRINCIPAL INVESTIGATOR: Brian H. Ladle
Elizabeth M. Jaffee, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21205

REPORT DATE: August 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Annual Summary (Jul 15, 2002 - Jul 14, 2003)

4. TITLE AND SUBTITLE
Dissecting the Mechanisms of T Cell Tolerance for More Effective Breast Cancer Vaccine Development

5. FUNDING NUMBERS
DAMD17-01-1-0282

6. AUTHOR(S)
Brian H. Ladle
Elizabeth M. Jaffee, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
John Hopkins University
Baltimore, MD 21205

E-Mail: bladle@jhmi.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

T cell tolerance to tumor-associated antigens is a significant barrier to immune based treatments of human cancers. One such tumor-associated antigen is the proto-oncogene *HER-2/neu* (*neu*) which is overexpressed in 35-40% of all human breast cancers. Although patients with neu expressing tumors develop antibody and T cell responses to this antigen, these responses are weak and unable to hinder tumor growth. Our work has focused on understanding these mechanisms of T cell tolerance using the *neu*-N transgenic mice that express the wild type rat *neu* cDNA under control of the MMTV promoter. Since neu is an endogenously expressed antigen, profound neu-specific immune tolerance exists in the *neu*-N mice. We previously reported the immunodominant T cell epitope of neu recognized by parental FVB/N mice, $RNEU_{420-429}$. We have investigated whether altering $RNEU_{420-429}$ can generate a more immunogenic peptide that will result in better protection from a HER-2/neu expressing tumor in the *neu*-N mice. Also, using GFP-expressing $RNEU_{420-429}$-specific T cells, we demonstrate that high avidity CTL cannot persist in the periphery of *neu*-N mice but do persist in the periphery of parental mice. Further studies are underway to understand the role other immune cells (such as $CD4^+CD25^+$ regulatory T cells) play in $CD8^+$ T cell tolerance. This work to further understand the mechanisms of T cell tolerance in this cancer model should lead to even further improvements in vaccination strategy for cancer immunotherapies.

14. SUBJECT TERMS
Immunology, murine T cell tolerance, cancer immunotherapy

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Brian H. Ladle
Phone: 410-614-1772
Fax: 410-614-8216

Address:
CRB 4M06, 1650 Orleans Street
Baltimore, MD 21231

Introduction

In humans, the proto-oncogene *HER-2/neu* (*neu*) is overexpressed in 35-40% of all breast cancers (1). Although patients with neu expressing tumors develop antibody and T cell responses to this antigen, these responses are weak and unable to hinder tumor growth. This suggests that T cell tolerance is a significant barrier to immune based treatments that target neu and most likely other antigens expressed by breast cancers. *neu*-N transgenic mice express the wild type rat *neu* cDNA under control of the MMTV promoter (2). Female mice spontaneously develop focal mammary tumors which overexpress the transgene in a stochastic manner starting approximately at 4 months of age. Since neu is an endogenously expressed antigen, it is likely that neu-specific immune tolerance exists in the *neu*-N mice. Three findings strongly support the existence of neu-specific tolerance in these mice (3). First, subcutaneous injection of 100-fold less transplantable *neu*-expressing mammary tumors is required for tumor growth in the *neu*-N mice when compared with the parental FVB/N mice. Second, neu-specific vaccines designed to prevent tumor development provide long-term prevention of *neu*-expressing tumors in the FVB/N mice but only delay slightly tumor growth in the *neu*-N mice. Third, neu-specific $CD8^+$ T cells (CTL) derived from FVB/N mice can lyse neu-expressing mammary tumors much more effectively than T cells derived from *neu*-N mice. Based on these findings, the *neu*-N transgenic mouse model of mammary tumors provides a clinically relevant model for developing vaccines that overcome tumor tolerance. The overall aim of this work is to determine the mechanism(s) by which immune tolerance of CTL inhibits effective immunization in the *neu*-N transgenic mice.

Body

An amino acid substitution in neu peptide epitope generates more immunogenic peptide

We previously reported the identification of $RNEU_{420\text{-}429}$ (PDSLRDLSVF) as the immunodominant epitope in rat neu restricted to the MHC class I molecule $H\text{-}2D^q$. This data was used to generate a MHC/peptide tetramer, and this reagent was used to compare the avidities of T cells derived from neu-vaccinated FVB/N mice to those from *neu*-N mice. We discovered that T cells from *neu*-N mice were low avidity as compared with T cells from FVB/N mice, which explains in part the tolerance to neu in *neu*-N mice.

To try to overcome this tolerance, we searched for altered peptide analogues of the immunodominant neu peptide that had enhanced immunogenicity in our system. Altered forms of $RNEU_{420\text{-}429}$ were created by substituting alanine at each of the 10 positions. This type of approach has been successful in identifying heteroclitic T cell peptides in both rodent and human settings (4). In the majority of cases, substitutions did not enhance recognition. However, when alanine was substituted for glutamate at position 2, this peptide (designated $RNEU_{420\text{-}429}A2$) demonstrated markedly improved recognition by a FVB/N-derived T cell clone in a lysis assay as compared with wild-type peptide (**figure 1**).

To determine if this heteroclitic peptide can immunize mice against mammary tumor expressing the natural $RNEU_{420\text{-}429}$ epitope, both wild-type $RNEU_{420\text{-}429}$ and the heteroclitic variant $RNEU_{420\text{-}429}A2$ were used to vaccinate mice. Dendritic cells derived from FVB/N mice were pulsed *in vitro* with either of these peptides (or with an irrelevant peptide) and then injected subcutaneously into FVB/N and *neu*–N mice followed by a subcutaneous challenge with the neu-

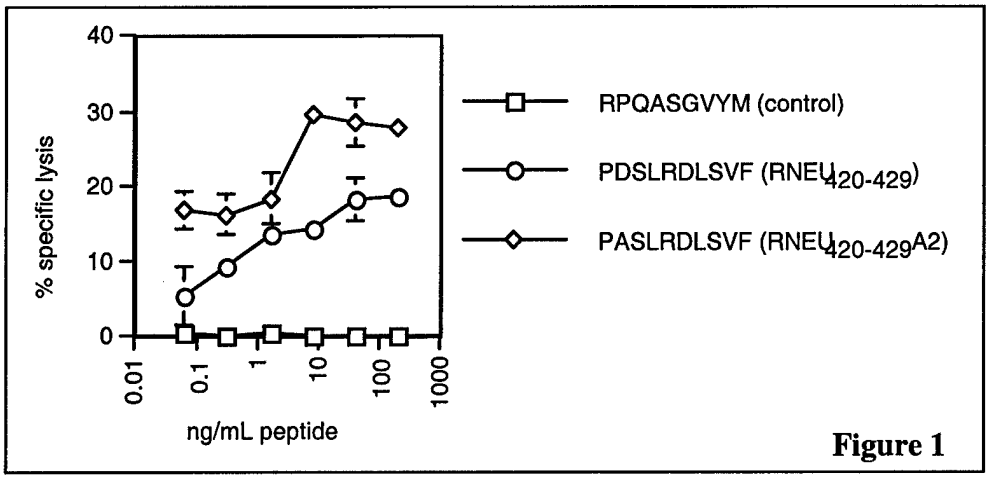

Figure 1

expressing mammary tumor line NT2. As shown in **Figure 2**, mice immunized with dendritic cells pulsed with the wild-type peptide developed tumor at about the same rate as mice immunized with an irrelevant peptide ($p<0.15$ for FVB/N mice, $p<0.39$ for *neu*-N mice). However, FVB/N mice immunized with the heteroclitic peptide showed a lag in tumor growth as compared to FVB/N mice immunized with the irrelevant peptide (**A,** $p<0.012$). Although not statistically significant, *neu*-N mice demonstrated a promising trend toward protection when vaccinated with the heteroclitic peptide (**B,** $p<0.21$).

Following the survival of transferred neu-specific T cells in vivo

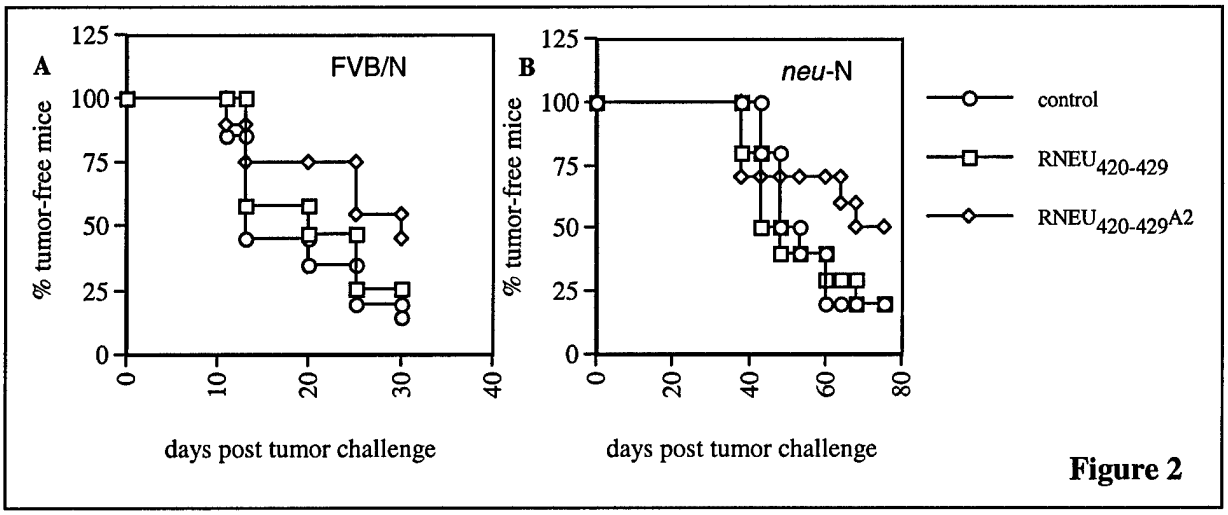

Figure 2

We had shown previously that transfer of a high-avidity $RNEU_{420-429}$-specific parental CTL line into mice given a neu-expressing mammary tumor inoculation provided protection from tumor outgrowth in FVB/N mice but not *neu*-N mice, suggesting that high-avidity neu-specific CTL are deleted or anergized in the periphery of transgenic mice. We had originally proposed to track the fate of neu-specific T cells adoptively transferred into both FVB/N and *neu*-N mice using a MHC/peptide dimer. However, no $RNEU_{420-429}$–specific T cells could be detected in the peripheral organs of mice via flow cytometry staining using either this molecule or the tetramer. The reason for this is not known definitively. Therefore, the fluorescent molecule green fluorescent protein (GFP) was stably transfected via the retrovirus RV-A6.MGIN (5) into the above mentioned CTL lines so that it could be easily detected in the FL1 channel in flow cytometry assays. These GFP-expressing T cells were transferred into FVB/N and *neu*-N mice (**figure 3**). At 3 and 48 hours post T cell transfer, mice were sacrificed and spleens ficolled and washed in FACS buffer prior to FACS analysis. While GFP-positive T cells are detectable in the spleens of both FVB/N and *neu*-N mice at 3 hours (**A** and **B**), after 48 hours they are only detectable in FVB/N mice (**C** and **D**). The inability of high-avidity neu-specific CTL to persist

in the periphery of *neu*-N mice suggests that these CTL are either being actively deleted or else do not receive necessary survival signals in this environment.

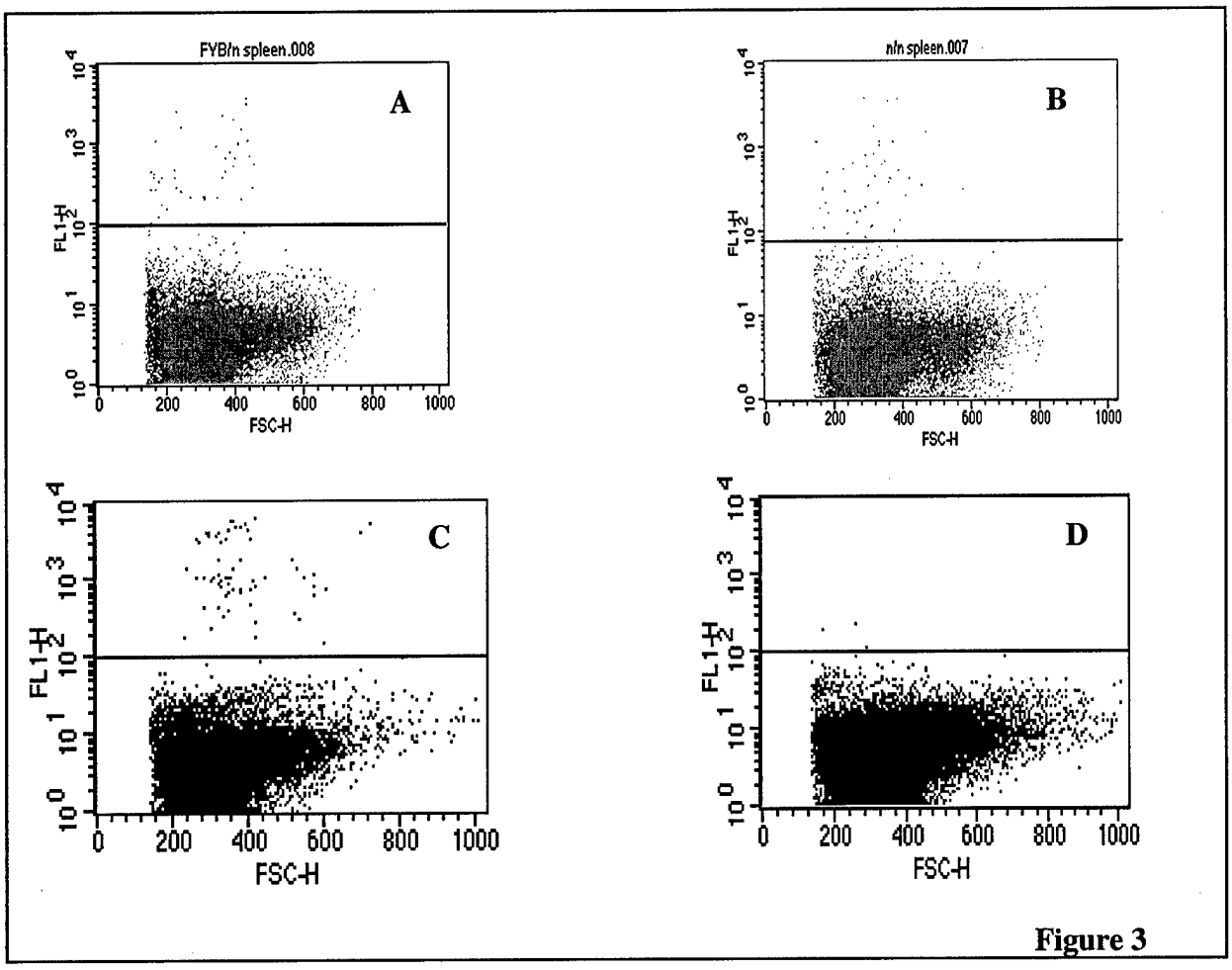

Figure 3

Key Research Accomplishments

- Generated altered, more immunogenic version of previously identified immunodominant MHC Class I epitope in rat neu ($RNEU_{420-429}$→$RNEU_{420-429}$A2).
- Demonstrated that vaccination of both parental and transgenic mice with the altered peptide resulted in improved tumor protection over vaccination with wild-type version.
- Used GFP-expressing $RNEU_{420-429}$-specific T cells to demonstrate that high avidity CTL cannot persist in the periphery of transgenic mice but do persist in the periphery of parental mice.

Reportable Outcomes

- **Ercolini, A.M.**, Machiels, J.P., Chen, Y.C., Slansky, J.E., Giedlen, M., Reilly, R.T., and Jaffee, E.M. Identification and Characterization of the Immunodominant Rat HER-2/neu MHC Class I Epitope Presented by Spontaneous Mammary Tumors from HER-2/neu-Transgenic Mice. *The Journal of Immunology*, 2003, 170: 4273-4280.

- **Ercolini, A.M., Ladle, B.H.**, Reilly, R. T., and Jaffee, E. M. Peripheral anergy of high avidity $CD8^+$ T cells specific for the immunodominant rat HER-2/neu epitope explains one mechanism of $CD8^+$ T cell tolerance. (manuscript in preparation)
- **Ercolini, A.M.**, Armstrong, T., **Ladle, B.H.**, Machiels, J.P., Lei, R.Y., Reilly, R.T. and Jaffee, E.M. An alternate, lower-affinity neu-specific T cell repertoire in HER-2/neu transgenic mice relative to the parental strain may explain neu-specific tolerance to neu-expressing tumors. Keystone Symposia on Basic Aspects of Tumor Immunology, Keystone, CO, Feb. 2003. Abstract #318. Poster Presentation.
- **Ladle, B.H.**, Manning, E.M., Emens, L.A., **Ercolini, A.M.**, Machiels, J.P., Jaffee, E.M. Reversal of $CD8^+$ peripheral tolerance in the HER-2/neu transgenic mice by deletion of $CD4^+CD25^+$ regulatory T cells. Keystone Symposia on Basic Aspects of Tumor Immunology, Keystone, CO, Feb. 2003. Abstract #333. Poster Presentation.
- **Ercolini, A.M.**, Armstrong, T.D., **Ladle, B.H.**, Machiels, J.P., Lei, R., Reilly, R.T., and Jaffee, E.M. Presentation at New Investigators' Luncheon entitled "Dissecting the mechanisms of T cell tolerance for more effective breast cancer vaccine development." Department of Defense Breast Cancer Research Program Meeting, Orlando, FL, September 28, 2002. Abstract#P54-12.
- Doctorate of Philosophy Degree in Immunology from The Johns Hopkins University School of Medicine awarded to **Anne M. Ercolini** in February 2003.
- GFP-expressing $RNEU_{420-429}$-specific T cell line
- Post-Doctoral Research Fellow – Position obtained by **Anne M. Ercolini** in March 2003 in the laboratory of Stephen D. Miller, Department of Microbiology-Immunology and Interdepartmental Immunobiology Center, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, IL 60611

Conclusions

Although the presence of antigen-specific CTL has been demonstrated in patients with tumor, the responses for the most part are weak and unable to hinder the growth of the malignancy. This has been shown in many types of cancer including neu-specific T cells in breast cancer. In some instances this may be due to an ineffective vaccine approach. However, in many cases mechanisms of peripheral T cell tolerance to specific tumor antigens may be at work. A major goal of cancer research is to develop therapies that will reverse the tolerant state and allow T cells to more effectively respond to tumor. *neu*-N transgenic mice display tolerance to the endogenous *neu* transgene and are therefore a clinically relevant model of breast cancer. The aim of this proposal is to dissect the mechanisms of $CD8^+$ T cell tolerance in this mouse model. Knowledge of the mechanisms of tolerance in these mice will lead to the development of more effective vaccine strategies that can overcome tolerance to the neu antigen.

We previously identified $RNEU_{420-429}$ as the immunodominant epitope in rat neu, and that vaccinated *neu*-N mice develop low-avidity CTL specific for this peptide as compared with CTL from FVB/N mice. One major goal is to develop vaccine strategies that will allow transgenic mice to develop high-avidity neu-specific T cells. For some antigens, altering the wild-type MHC I epitope so that it binds more strongly to MHC and/or demonstrates greater recognition *in vitro* by antigen specific T cells improves the immunization potential and the clinical outcome (6). Here, we show that vaccinating FVB/N and *neu*–N mice with dendritic cells pulsed with a heteroclitic variant of the wild-type epitope also induces improved protection against tumors that express the natural $RNEU_{420-429}$ epitope as compared with immunization with the $RNEU_{420-429}$ epitope itself. Further study needs to be done to determine if improvement in tumor-free survival

is due to the generation of higher-avidity CTL in *neu*-N mice vaccinated with the heteroclitic peptide. Although this regimen is so far less efficacious than the vaccination strategies we have developed using the entire neu protein, it is hoped that further study will build upon the principles gleaned from these experiments and lead to improvements relevant to treatment strategies in patients.

We showed previously that high avidity $RNEU_{420-429}$ specific T cells derived from parental mice could cure parental mice of transplanted neu-expressing tumor but could not cure transgenic mice. We hypothesized that this was due to the anergy or deletion of high avidity T cells in *neu*-N mice and wanted to track their fate in the periphery of mice after transfer. Using a GFP-expressing CTL line derived from FVB/N mice, we showed that high avidity $RNEU_{420-429}$-specific T cells persist in the periphery of FVB/N mice but not *neu*-N mice.

Further studies are underway to understand the role other immune cells play in this $CD8^+$ T cell tolerance. Initial experiments indicate that $CD4^+CD25^+$ regulatory T cells from *neu*-N mice are able to suppress $RNEU_{420-429}$ specific T cells from FVB/N mice. Other initial experiments show that depletion of $CD4^+CD25^+$ regulatory T cells in *neu*-N mice using the monoclonal antibody PC61 (rat IgG specific to mouse CD25) increases the number of $RNEU_{420-429}$ specific T cells that can be detected following a HER-2/neu targeted vaccine.

In summary, it is likely that further investigation of the mechanism of T cell tolerance in this cancer model will lead to even further improvements in vaccination strategy for the eradication of tumors.

References

1. Disis, M. L. and Cheever, M. A. HER-2/neu protein: a target for antigen-specific immunotherapy of human cancer, Adv Cancer Res. *71:* 343-71, 1997.
2. Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease, Proc Natl Acad Sci U S A. *89:* 10578-82, 1992.
3. Reilly, R. T., Gottlieb, M. B., Ercolini, A. M., Machiels, J. P., Kane, C. E., Okoye, F. I., Muller, W. J., Dixon, K. H., and Jaffee, E. M. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice, Cancer Res. *60:* 3569-76., 2000.
4. Slansky, J. E., F. M. Rattis, L. F. Boyd, T. Fahmy, E. M. Jaffee, J. P. Schneck, D. H. Margulies, and D. M. Pardoll. 2000. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity 13:529.*
5. Tuting, T., J. Steitz, J. Bruck, A. Gambotto, K. Steinbrink, A. B. DeLeo, P. Robbins, J. Knop, and A. H. Enk. 1999. Dendritic cell-based genetic immunization in mice with a recombinant adenovirus encoding murine TRP2 induces effective anti-melanoma immunity. *J Gene Med 1:400.*
6. Hoffmann, T. K., D. J. Loftus, K. Nakano, M. J. Maeurer, K. Chikamatsu, E. Appella, T. L. Whiteside, and A. B. DeLeo. 2002. The ability of variant peptides to reverse the nonresponsiveness of T lymphocytes to the wild-type sequence p53(264-272) epitope. *J Immunol 168:1338.*

APPENDIX COVER SHEET

Identification and Characterization of the Immunodominant Rat HER-2/neu MHC Class I Epitope Presented by Spontaneous Mammary Tumors from HER-2/neu-Transgenic Mice[1]

Anne M. Ercolini, Jean-Pascal H. Machiels,[2] Yi Cheng Chen, Jill E. Slansky,[3] Martin Giedlen,[4] R. Todd Reilly, and Elizabeth M. Jaffee[5]

The HER-2/neu (*neu*-N)-transgenic mice are a clinically relevant model of breast cancer. They are derived from the parental FVB/N mouse strain and are transgenic for the rat form of the proto-oncogene HER-2/neu (*neu*). In this study, we report the identification of a MHC class I peptide in the neu protein that is recognized by CD8$^+$ T cells derived from vaccinated FVB/N mice. This 10-mer was recognized by all tumor-specific FVB/N T cells generated regardless of the TCR Vβ region expressed by the T cell or the method of vaccination used, establishing it as the immunodominant MHC class I epitope in neu. T cells specific for this epitope were able to cure FVB/N mice of transplanted neu-expressing tumor cells, demonstrating that this is a naturally processed peptide. Altered peptide analogs of the epitope were analyzed for immunogenicity. Vaccination with dendritic cells pulsed with a heteroclitic peptide provided FVB/N and *neu*-N mice with increased protection against tumor challenge as compared with mice immunized with dendritic cells loaded with either wild-type or irrelevant peptide. Discovery of this epitope allows for better characterization of the CD8$^+$ T cell responses in the *neu*-N mouse model in which neu-specific tolerance must be overcome to produce effective antitumor immunity. *The Journal of Immunology,* 2003, 170: 4273–4280.

An important role for Ag-specific CD8$^+$ CTLs in antitumor immunity has been demonstrated in both murine tumor models and cancer patients. In mice, immunization with model and naturally expressed tumor Ags or their MHC class I (MHC-I)[6] epitopes induces antitumor CTL that mediate in vivo tumor rejection (1–12). Patients with established disease have shown some response when given adoptively transferred tumor-specific CTL (13–15). Similarly, clinical trials testing the efficacy of immunizing with MHC-I tumor epitopes from the human papilloma virus Ag E7 have resulted in T cell responses in some patients with cervical cancer (16–18). Several trials undertaken with peptides derived from melanoma-associated Ags have resulted in the induction and expansion of Ag-specific CTL. In some cases, immunization was also associated with clinical responses (19–22). However, in the majority of patients, immunization with MHC-I peptides induces T cell responses that are weak and ineffective in inducing significant tumor regression. As with all cancer therapies, the application of spontaneously arising mouse cancer models is central to the development of enhanced immunotherapies for human cancer (23).

We previously described *neu*-N-transgenic mice as a model of breast cancer that closely mimics immune tolerance described in some patients with cancer (24). These mice, derived from the parental FVB/N strain, express the wild-type rat HER-2/neu (*neu*) cDNA under the control of a mouse mammary tumor virus promoter (25). Female mice spontaneously and stochastically develop mammary tumors beginning at 4 mo of age. Mammary glands that have become tumorigenic overexpress the *neu* transgene relative to *neu* expression in normal glands in the same mice. Because the neu tumor Ag is endogenous to the host, this allows for the development of tolerance to the Ag, as evidenced by the poor ability of these mice to develop neu-specific antitumor immunity following vaccination as compared with the parental FVB/N mice. In the parental strain, depletion of CD8$^+$ T cells before neu-specific vaccination inhibits their ability to reject a subsequent tumor challenge (24). Similarly, depletion of CD8$^+$ T cells in transgenic mice before vaccination accelerates tumor outgrowth (26–29). However, unlike the parental mice, the transgenic mice are rarely cured of neu-expressing tumors. These data suggest that neu-specific CTL in *neu*-N mice are either weak lytic agents or are actively tolerized, either through deletion or anergy induction among high-avidity T cells.

The current study was designed to identify epitopes expressed by the rat neu and recognized by FVB/N (H-2^q)-derived CTL.

Department of Oncology, Sidney Kimmel Cancer Center at Johns Hopkins, Graduate Program in Immunology, The Johns Hopkins School of Medicine, Baltimore, MD 21231

Received for publication July 18, 2002. Accepted for publication February 13, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health/National Cancer Institute National Cooperative Drug Discovery Group Grant 2U19CA72108 (to E.M.J.), Specialized Programs of Research Excellence in Breast Cancer Grant 1P50CA88843-01 (to E.M.J.), Breast Cancer Research Foundation Grant (to E.M.J.), American Cancer Society Research Scholar Grant RSG-01-080-01-LIB (to R.T.R.), Susan G. Komen Foundation Grant BCTR00-00068 (to R.T.R.), National Institutes of Health Grant CA76669 (to J.E.S.), Cancer Research Institute Grant 311-2007 (to A.M.E.), National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant 5T32AI07247-21 (to A.M.E.), and Department of Defense Grant DAMD17-01-1-0282 (to A.M.E.). J.P.M. is a Fulbright Scholar supported by a grant from Belgium Televie-Fonds National de la Recherche Scientifique (credit 7.4568.98) and Oeuvre Belge du Cancer.

[2] Current address: Laboratoire d'Oncologie Experimentale, Avenue Hippocrate, 54, UCL 5471, Tour Claude Bernard 4-ieme Etage, B-1200 Brussels, Belgium.

[3] Current address: Integrated Department of Immunology, National Jewish Medical and Research Center, 1400 Jackson Street, Room K511a, Denver, CO 80206.

[4] Current address: Cerus Corporation, 2411 Stanwell Drive, Concord, CA 94520.

[5] Address correspondence and reprint requests to Dr. Elizabeth Jaffee, Department of Oncology, Sidney Kimmel Cancer Center at Johns Hopkins, Bunting-Blaustein Cancer Research Building 4 M07, 1650 Orleans Street, Baltimore, MD 21231. E-mail address: ejaffee@jhmi.edu

[6] Abbreviations used in this paper: MHC-I, MHC class I; HA, hemagglutinin; ICS, intracellular cytokine staining.

0022-1767/03/$02.00

Identification and characterization of these epitopes are critical to understanding the difference in neu-reactive T cell responses between the transgenic and parental mice. Numerous tolerance studies have shown that T cells reactive with endogenous Ags expressed in the thymus are deleted (30–33), although some T cells can escape and exist in the periphery in a functionally unresponsive state (30, 34, 35). Similarly, T cells reactive with endogenous Ags not expressed in the thymus may be deleted or rendered nonresponsive in the periphery (36–38). Knowledge of the MHC-I epitopes in neu will allow us to study the fate of T cell responses directed at these epitopes in the *neu*-N-transgenic tolerant mice. In addition, it may be possible to alter these epitopes to identify heteroclitic epitopes with improved immunogenicity in the transgenic mice.

We have identified the immunodominant MHC-I epitope in the rat neu protein. This peptide is recognized by all neu-specific T cell lines and clones we derived from the splenocytes of vaccinated FVB/N mice, regardless of the TCR Vβ region expressed. In addition, we show that adoptive transfer of T cells specific for this peptide is protective in vivo. Furthermore, immunization with a heteroclitic version of this peptide is also protective against challenge with mammary tumors expressing the naturally processed epitope.

Materials and Methods

Peptides and primers

A panel of 135 peptides from fragment 4 (see Fig. 2) used in the initial screening were synthesized by Chiron (Emeryville, CA). All other peptides (>95% purity) were from the Johns Hopkins Biosynthesis and Sequence Facility (Department of Biochemistry, Johns Hopkins School of Medicine, Baltimore, MD). The PCR primers used to create the *neu* fragments (see Fig. 1) are as follows: fragment 1 (bp 1–508), 5′-CCGGGCCGAATTC GCAATGATC and 3′-CCCCGAATTCCTACTGAGGGTTCCCACGGA TCAA; fragment 2 (bp 458–886), 5′-GACATGAAGTTGCGGCTCC CTAGTCTCACAGAGATCCTGAAG and 3′-CCCCGAATTCCTACTC AGGGTTGTGCATGGACTC; fragment 3 (bp 836-1294), 5′-GACATGA AGTTGCGGCTCCCTGCCCTCGTCACCTACAACACA and 3′-CCCC GAATTCCTAGAGGTCACGGAGACTGTCTGG; fragment 4 (bp 1244–1675), 5′-GACATGAAGTTGCGGCTCCCTATCACAGGTTACCTGTA CATC and 3′-CCCCGAATTCCTACTTCCATACTCGGCACTCCTC; fragment 5 (bp 1607–2077), 5′-GACATGAAGTTGCGGCTCCCTAC CCAGTGTGTCAACTGCAGT and 3′-CCCCGGTACCCTAGATCTTC TGTCTCCTTCGTTT; fragment 6 (bp 2009–2476), 5′-GACATGAAGT TGCGGCTCCCTGGCGTCCTGCTGTTCCTGATC and 3′-CCCCGGTA CCCTAACCTCGGTGTTCTCGGACATG; fragment 7 (bp 2405–2872), 5′-GACATGAAGTTGCGGCTCCCTTCCACAGTACAGCTGGTGACA and 3′-CCCCGGTACCCTAGCAGATTGGAGGCTGAGGTAG; fragment 8 (bp 2801–3271), 5′-GACATGAAGTTGCGGCTCCCTGATGG AATCCCAGCCCGGGAG and 3′-CCCCGGTACCCTACCCTTCC GAGGGAGCCAGTGG; and fragment 9 (bp 3203–3796), 5′-GACATGAAGTTGCGGCTCCCTGAGCTGACACTGGGCCTGGAG and 3′-CCCCGGTACCCTATACAGGTACATCCAGGCCTAG. Each fragment was ligated into the pcDNA3.1 mammalian transfection vector (Invitrogen, Carlsbad, CA) at the multicloning site.

Cell lines and medium

The IT22 cell line derives from a spontaneously transformed mouse fibroblast line as described previously (39). NIH 3T3 cells are a mouse fibroblast line (ATCC CRL-1658; American Type Culture Collection (ATCC), Manassas, VA). Rat *neu* cDNA was cloned from pSV2-*neu*-N (40) and ligated into the pcDNA3.1 vector (Invitrogen). IT22 cells were transfected with this construct by electroporation (20 μg/10^7 cells) to produce IT22neu cells. The human *B7-1* gene was retrovirally inserted into rat neu-expressing NIH 3T3 cells (3T3neu; ATCC CRL-1915) using previously described methods (41) to produce 3T3neuB7-1 cells. The same neu-expressing NIH 3T3 cells were transduced with a murine GM-CSF retrovirus using the same methods for inserting the *B7-1* gene to create 3T3neuGM vaccine cells. L-D^q cells and L-L^q cells are murine fibroblast cell lines transfected with murine H-$2D^q$ or -L^q, respectively (42). All of the above cell lines were maintained at 37°C and 10% CO_2 in DMEM (Life Technologies, Rockville, MD) supplemented with 10% bovine calf serum (HyClone, Logan, UT), 1% L-glutamine (JRH Biosciences, Lenexa, KS), 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO), 1% sodium pyruvate (Sigma-Aldrich), and 0.5% penicillin/streptomycin (Sigma-Aldrich). The NT2 and NT5B7-1 neu-expressing tumor lines are derived from spontaneous mammary tumors excised from the *neu*-N mice as previously described (24, 43).

Cloning and transfection of H-2D^q

Murine H-$2D^q$ was cloned from the L-D^q cell line (5′ primer, ATGGCTC CGCGCACGCTGCT; 3′ primer, TCACGCTTTACAATCTCGGA) and ligated into pcDNA3.1. The T2 cell line is a B lymphoblast/T lymphoblast hybrid human cell line deficient in the MHC-I TAP transporter molecule (ATCC CRL-1992). These cells were transfected with D^q by electroporation (20 μg/1 × 10^7 cells) to create the T2D^q cell line. T2D^q cells were maintained at 37°C and 5% CO_2 in RPMI 1640 (Life Technologies) supplemented with 10% FBS (HyClone), 1% L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 0.5% penicillin/streptomycin.

neu-specific T cell lines and clones and hemagglutinin (HA)-specific T cell line

Some $CD8^+$ T cell lines were derived from FVB/N mice that were s.c. vaccinated with 1 × 10^6 irradiated 3T3neuGM cells at each of three sites (two forelimbs and one hindlimb). Mice were sacrificed, and spleens were excised 2 wk later. Splenocyte cultures were initially stimulated every 5 days with irradiated, IFN-γ-treated NT5B7-1 cells and then every 9 days by the addition of irradiated 3T3neuB7-1 cells as stimulators and FVB/N-derived splenocytes as feeders. Clones were developed from this line by limiting dilution. Other $CD8^+$ T cell lines were produced from the splenocytes of mice vaccinated with a neu-expressing recombinant vaccinia or neu plasmid DNA as described previously (24). A T cell line specific for the irrelevant Ag HA was derived from mice vaccinated with HA recombinant vaccinia virus as described previously (44). T cells were maintained at 37°C and 5% CO_2 in CTL medium (RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 0.1% 2-ME (Sigma-Aldrich), and 0.5% penicillin/streptomycin) supplemented with 10 cetus U/ml murine IL-2 (supernatant from B16 IL-2 line (45)).

Chromium release assays

Lysis assays were performed in triplicate in 96-well V-bottom plates as previously described (24). Briefly, target cells were resuspended in 100 μl of CTL medium and labeled with 0.2 mCi of ^{51}Cr/2 × 10^6 cells at 37°C and 5% CO_2 for 1 h. Cells were washed in CTL medium and resuspended at 6 × 10^4 cells/ml in RPMI 1640. To pulse peptide onto targets, 100 μl of peptide in RPMI 1640 was added to 50-μl targets for 1 h at room temperature in each well. After the removal of 100 μl of supernatant, 150 μl of T cells in CTL medium was added for the indicated E:T ratio. After a 4-h incubation, 100 μl of supernatant was assayed for ^{51}Cr release and percent specific lysis was determined by the formula: ((^{51}Cr release sample − spontaneous ^{51}Cr release target alone)/(maximum ^{51}Cr release target alone − spontaneous ^{51}Cr release target alone)) × 100. For the Ab blocking assays, the H-$2D^q$ Ab 30-5-7S (ATCC HB-31) was added for 30 min at 37°C to 100 μl of target cells resuspended in CTL medium at a final concentration of 50 μM before addition of effector T cells.

Development and transfection of neu fragments

Nine overlapping fragments of the *neu* cDNA were created using specific primers and PCR amplification (Fig. 1*A*) and then ligated into pcDNA3.1. The fragment constructs were then transfected into NIH 3T3 cells using electroporation (20 μg/1 × 10^7 cells) or Lipofectamine (1.5 μg/3 × 10^5 cells; Life Technologies). Limiting dilution produced several clones of each fragment. RT-PCR was performed using primers described above to determine which clones expressed fragment mRNA.

Mice and dendritic cell immunizations

FVB/N mice were purchased at 6–8 wk of age from the National Cancer Institute (Bethesda, MD). *neu*-N mice (25) were bred to homozygosity as verified by Southern blot analysis. Splenic dendritic cells were generated from FVB/N mice as previously described (46). On the day of immunization, cells were collected, resuspended at 4 × 10^6/ml in AIM-V medium (Life Technologies), and pulsed with 300 μg/ml peptide for 3 h. Cells were then washed three times in HBSS (pH 7.4) and resuspended at 2 × 10^6 cells/ml. Mice were given 0.1 ml s.c. injections in each hindlimb on days 0 and 7 and challenged with NT2 mammary tumor cells in the right hindlimb on day 14.

GM-CSF release ELISA

Peptides were pulsed onto NIH 3T3 cells (which were grown overnight at 26°C to increase the number of empty MHC on the cell surface) in RPMI 1640 at 26°C for 1 h at a final concentration of 0.1 μM. Targets were then

washed twice in CTL medium, and 1×10^5 T cells were added for a 1:1 E:T ratio. Plates were incubated at 37°C and 5% CO_2 for 24 h, and supernatants were harvested for the ELISA (Endogen, Woburn, MA). For the screening of *neu* fragments, 1×10^5 T cells and 3×10^5 targets were plated in CTL medium for 24 h and an ELISA was performed.

Flow cytometry (FACS)

Cells were stained by washing them two times in FACS buffer (1× HBSS (pH 7.4), 2% FBS, 1% HEPES (Life Technologies), and 0.1% NaN_3 (Sigma-Aldrich)) and incubating with Ab for 20 min at 4°C. Staining of TCR Vβ4, Vβ14, and Vβ17 was done using supernatants from hybridomas KT4-10, 14-2, and KJ23, respectively (47). Abs to Vβ2, -6, and -7, and fluorescence-conjugated secondary Abs were purchased from BD PharMingen (La Jolla, CA). Ab to CD8 was purified from the 2.43 hybridoma (ATCC HB-27). MHC staining was done using supernatant collected from hybridomas 30-5-7S, 113, and 28-14-8S, which are specific for H-2D^q (42). The secondary Ab for all three Abs was FITC-conjugated goat anti-mouse IgG (BD PharMingen).

Intracellular cytokine staining (ICS)

ICS was performed as directed using a BD PharMingen kit for detection of murine IFN-γ. Briefly, 1×10^6 splenocytes (nylon wool purified to deplete B cells and macrophages) were incubated 12–16 h with an equal ratio of indicated targets in the presence of GolgiStop. Cells were washed in FACS buffer and stained with FITC-conjugated Ab to CD8, fixed and permeabilized, and stained with PE-conjugated Ab to IFN-γ.

Results

Generation of neu-specific CD8$^+$ T cells from vaccinated FVB/N mice

Three T cell lines were generated to characterize the T cell populations that result from different vaccination experiments as well as from different neu targeted vaccine approaches shown to be potent in other tumor models (48). A T cell line was therefore generated from mice vaccinated with the 3T3neuGM whole cell vaccine, and 11 neu-specific T cell clones were derived from this line. In addition, lines were created from mice vaccinated with the vaccinia vector expressing the entire neu protein and from mice vaccinated with neu plasmid DNA. All lines and clones were shown to lyse the full-length neu protein in a ^{51}Cr release assay using 3T3neu vs 3T3 wild-type targets (not shown). Analysis of the Vβ usage of these T cell lines and clones suggests that the neu CD8$^+$ T cell response is oligoclonal. Specifically, TCRs utilizing six Vβ regions (TCR Vβ2, -4, -6, -7, -14, and -17) were identified (data not shown).

Identification of RNEU$_{420-429}$, a peptide epitope contained in the extracellular domain of the rat neu protein, as the T cell target of all FVB/N-derived T cell lines and clones

As an initial approach to roughly map the position of neu epitopes, the *neu* cDNA was divided into nine approximately equal fragments overlapping by 15–25 aa (Fig. 1*A*). NIH 3T3 cells were

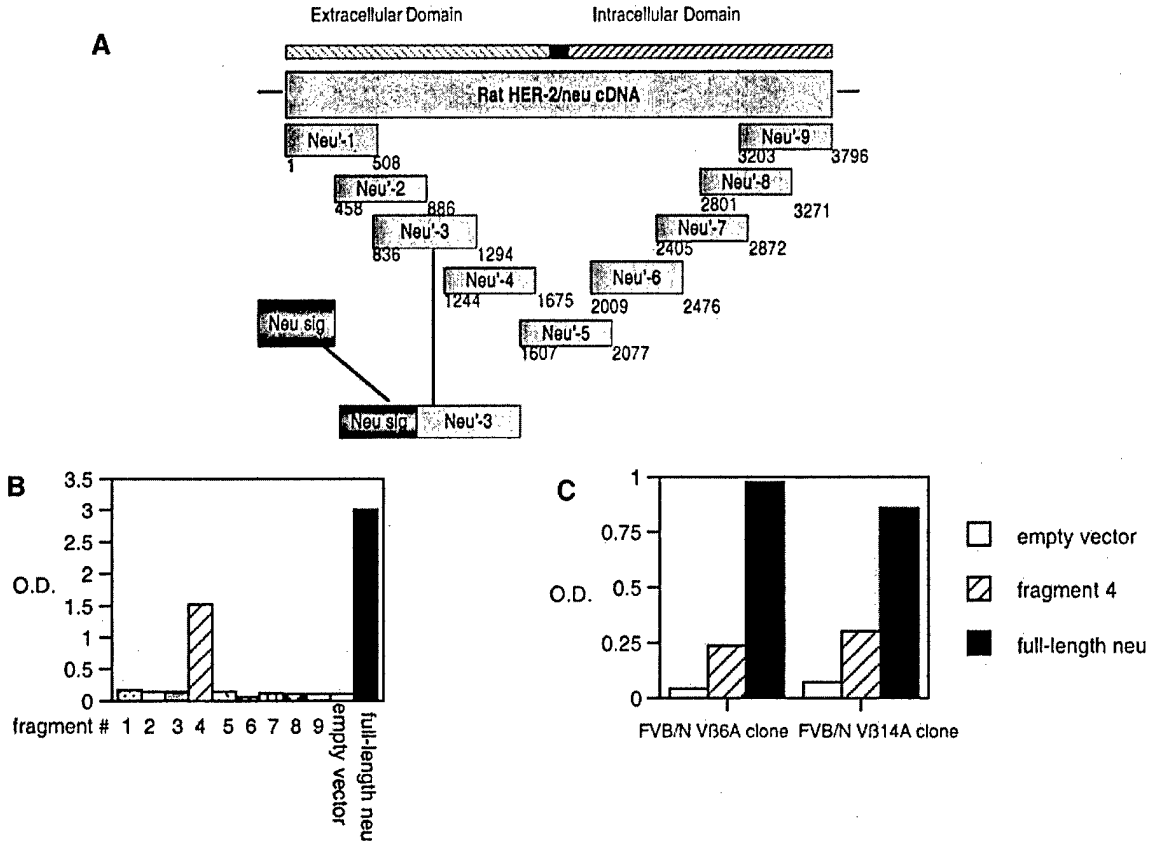

FIGURE 1. Extracellular fragment 4 of neu contains a neu MHC-I epitope recognized by the FVB/N-derived T cell clones. *A*, Nine overlapping fragments of *neu* cDNA were created using specific primers and PCR amplification. The numbers under each fragment refer to the starting and ending base pair within the entire *neu* cDNA. Each fragment was sequenced and confirmed to be the original rat *neu* sequence before ligation into the vector pcDNA3.1. The fragments were then transfected into 3T3 cells for use as targets in T cell assays. T cells (1×10^5) and 3×10^5 targets were plated in duplicate in 96-well plates and incubated at 37°C for 24 h. Supernatants were collected and tested for levels of GM-CSF by ELISA. Data are shown as degree of color change (OD). Effectors were FVB/N Vβ4A clone (TCR Vβ4) (*B*) and FVB/N Vβ6A clone (TCR Vβ6) and FVB/N Vβ14A clone (TCR Vβ14) (*C*). T cells show recognition of full-length neu (■) and of fragment 4 (▨).

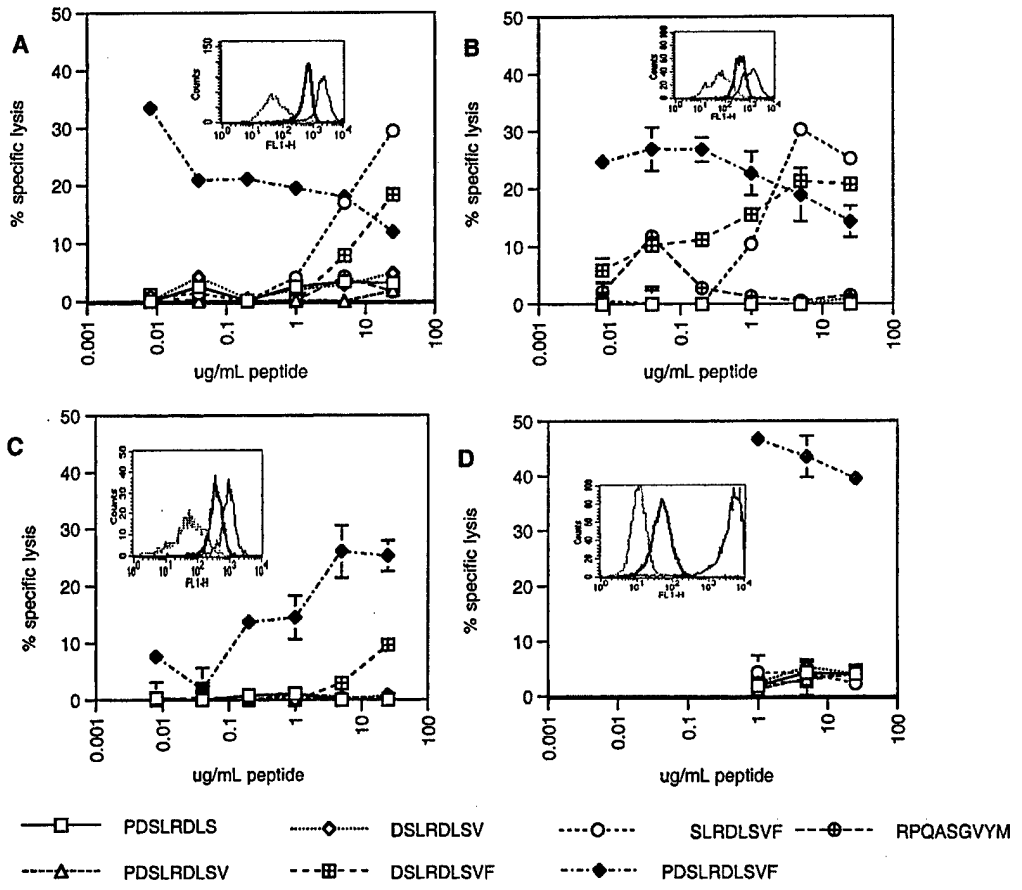

FIGURE 2. The original identified 10-mer $RNEU_{420-429}$ is the optimal epitope recognized by neu-specific T cell clones. Full-length and truncated versions of the epitope (as well as the irrelevant lymphocytic choriomeningitis virus nucleoprotein peptide $NP_{118-126}$ (68)) were pulsed onto 3×10^3 chromium-labeled IT22 cells for 1 h at 37°C. T cells were added before a 4-h incubation at 37°C. E:T ratios ranged from 15:1 to 25:1. Effectors were FVB/N Vβ2A clone (Vβ2) (*A*), FVB/N Vβ4A clone (Vβ4) (*B*), FVB/N Vβ6A clone (Vβ6) (*C*), and FVB/N Vβ14A clone (Vβ14) (*D*). *Insets*, Surface staining of T cell clones. Effectors were stained with Ab to CD8 (thin line), Ab to appropriate TCR Vβ region (thick line), or the irrelevant Ab rat IgG (dotted line).

transfected with these constructs and used as targets in GM-CSF release assays to identify which fragment contained the peptide(s) recognized by the clones. Fig. 1*B* shows recognition of 3T3 cells expressing fragment 4 (aa 410–553) by the FVB/N-derived T cell clone FVB/N Vβ4A (expressing TCR Vβ4). Recognition of fragment 4 was also seen with FVB/N Vβ6A clone (TCR Vβ6) and FVB/N Vβ14A clone (TCR Vβ14) (Fig. 1*C*). This fragment is located in the extracellular domain of neu near the transmembrane region.

Peptides 10 aa in length were synthesized, and these peptides spanned the entire sequence of fragment 4 (excluding the overlapping regions with fragments 3 and 5) and overlapped by nine residues each. Of the 135 peptides made, one ($RNEU_{420-429}$) was consistently recognized by clones in GM-CSF release ELISA (data not shown). This peptide (PDSLRDLSVF), along with its 8- and 9-aa derivatives, was synthesized and tested for recognition by neu-specific T cells in Cr^{51} release lysis assays. As shown in Fig. 2, the 10-mer was recognized more efficiently than the shorter peptides regardless of the TCR Vβ region expressed by the T cell. This peptide was subsequently found to be recognized by all T cell lines and clones derived, suggesting that $RNEU_{420-429}$ is the immunodominant MHC-I neu epitope recognized by FVB/N-derived T cells. To further analyze the $CD8^+$ T cell response to neu in FVB/N mice, frequency analysis was performed on the spleens of five individual mice given a s.c. 3T3neuGM vaccine. Fourteen days after vaccination, spleens were excised and cultured with 3T3*neu*B7-1 cells for 7 days before performing ICS to determine the response to full-length neu and to $RNEU_{420-429}$. As shown in Table I, all vaccinated FVB/N mice developed $CD8^+$ T cells that were specific for both full-length neu and for $RNEU_{420-429}$. This further supports the immunodominance of $RNEU_{420-429}$ in

Table I. *T cell specificity of vaccinated FVB/N and neu-N mice*[a]

Mouse No.	% neu Specific[b]	% $RNEU_{420-429}$ Specific[c]
FVB/N 1	1.83	2.48
FVB/N 2	0.88	1.92
FVB/N 3	1.47	2.75
FVB/N 4	0.06	0.48
FVB/N 5	0.28	1.16
neu-N 1	0.00	0.32
neu-N 2	0.13	0.22
neu-N 3	0.00	0.23
neu-N 4	0.00	0.00
neu-N 5	0.02	0.26
neu-N 6	0.00	0.07
neu-N 7	0.00	0.06
neu-N 8	0.16	0.00
neu-N 9	0.00	0.04
neu-N 10	0.14	0.00

[a] Specificity determined by the percentage of $CD8^+$ splenocytes staining positive for IFN-γ in response to target in ICS assay.

[b] neu specificity determined by the percentage responding to IT22neu minus the percentage responding to IT22.

[c] $RNEU_{420-429}$ specificity determined by the percentage responding to $T2D^q$ cells pulsed with $RNEU_{420-429}$ − the percentage responding to $T2D^q$ cells pulsed with the irrelevant peptide $NP_{118-126}$.

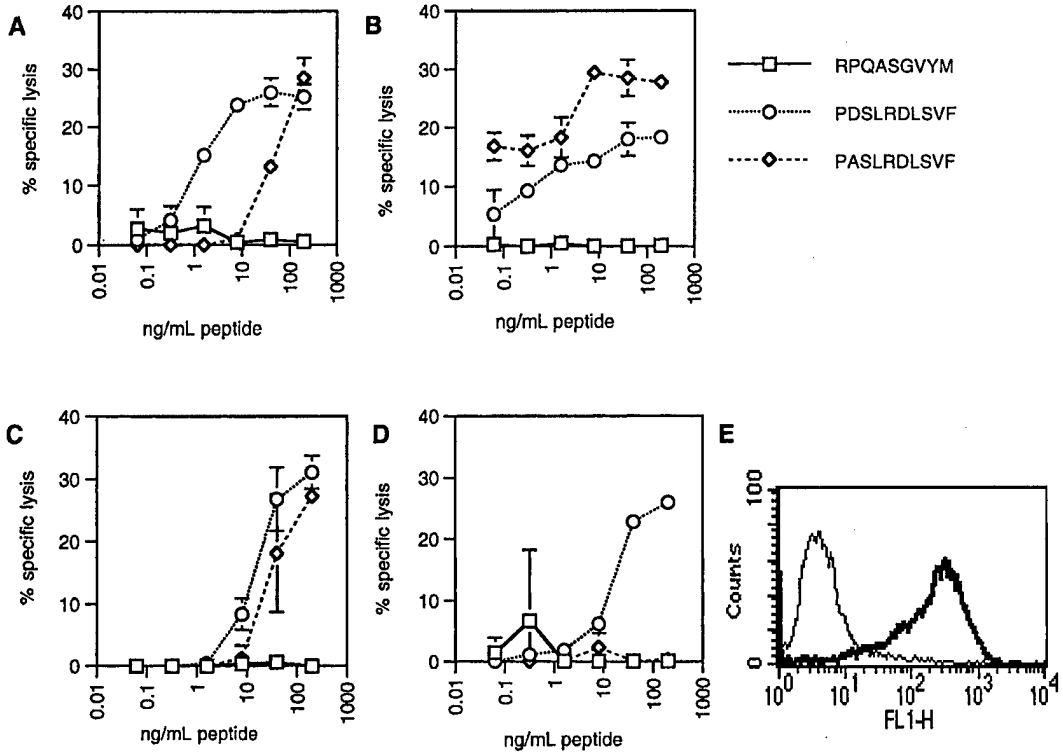

FIGURE 3. H-2D^q is the restricting MHC molecule for the recognition of $RNEU_{420-429}$ by FVB/N T cell clones. T2 cells were transfected via electroporation (20 $\mu g/10^7$ cells) with D^q cDNA to create T2D^q cells. Chromium-labeled T2D^q cells (3×10^3) were pulsed for 1 h with either $RNEU_{420-429}$ or the heteroclitic variant $RNEU_{420-429}$A2. T cells (3×10^4) were then incubated with the targets for 4 h at 37°C. Effectors were FVB/N Vβ2A clone (Vβ2) (*A*), FVB/N Vβ4A clone (Vβ4) (*B*), FVB/N Vβ6A clone (Vβ6) (*C*), and FVB/N Vβ14A clone (Vβ14) (*D*). T cell clone FVB/N Vβ4A demonstrates enhanced lysis of the $RNEU_{420-429}$ peptide with an alanine substituted at position 2 ($RNEU_{420-429}$A2). *E*, T2D^q cells were incubated with Ab to D^q (28-14-8S) followed by FITC-conjugated Ab to mouse IgG (thick line) to confirm surface expression of the MHC molecule (thin line, secondary Ab alone).

FVB/N mice. The same analysis was done on the spleens of 10 individual *neu*-N mice given the same vaccine. As shown in Table I, the tolerized *neu*-N mice do not demonstrate appreciable T cell activity for the immunodominant epitope.

H-2D^q is the restriction element for $RNEU_{420-429}$

Initial CTL blocking studies using an Ab specific for H-2D^q and -L^q (30-5-7S) indicated that lysis of neu was restricted to one of these two molecules. MHC restriction was further determined by pulsing $RNEU_{420-429}$ onto mouse L cells that were transfected with either H-2D^q or -L^q. FVB/N Vβ4A clone cells lysed targets transfected with D^q but not those transfected with L^q (data not shown). The D^q molecule was then cloned and transfected into T2 cells. When pulsed with $RNEU_{420-429}$, T2D^q cells were recognized in a ^{51}Cr release assay by all FVB/N-derived T cell lines and clones. Lysis of T2D^q cells pulsed with decreasing concentrations of the $RNEU_{420-429}$ peptide is shown in Fig. 3 for the four T cell clones also evaluated in Fig. 2. These data established D^q as the restricting MHC-I molecule for $RNEU_{420-429}$.

Adoptive transfer of $RNEU_{420-429}$-specific T cells into tumor-bearing FVB/N mice confirms that this peptide is a tumor rejection epitope naturally expressed by mammary tumor cells in vivo

FVB/N mice were given a s.c. leg injection of neu-expressing tumor cells (NT2) followed 1 day later by i.v. transfer of an FVB/N T cell line (derived from mice given neu plasmid vaccine) specific for $RNEU_{420-429}$. This line was chosen because it demonstrated the highest lysis of NT2 cells in vitro. As shown in Fig. 4, mice receiving these T cells showed protection from tumor outgrowth as compared with mice receiving a control T cell line specific for the irrelevant Ag HA ($p < 0.0008$). This experiment was repeated using several $RNEU_{420-429}$ specific T cell clones and lines with similar results.

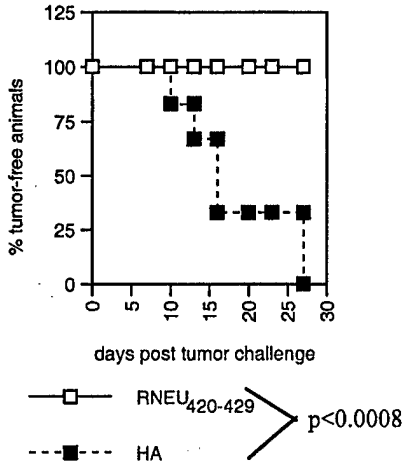

FIGURE 4. Confirmation that $RNEU_{420-429}$ is the naturally processed mammary tumor epitope expressed in vivo. FVB/N mice (9–10 mice/group) were given a s.c. leg injection of 5×10^6 neu-expressing tumor cells (NT2 cells derived from spontaneously arising mammary tumors in *neu*-N mice). One day later, mice were given an i.v. transfer of 1×10^7 $CD8^+$ T cells specific for either $RNEU_{420-429}$ or for the irrelevant Ag HA. Mice were observed twice a week for the development of tumor in the leg. Additional independent studies were performed with other T cell clones and lines with similar results.

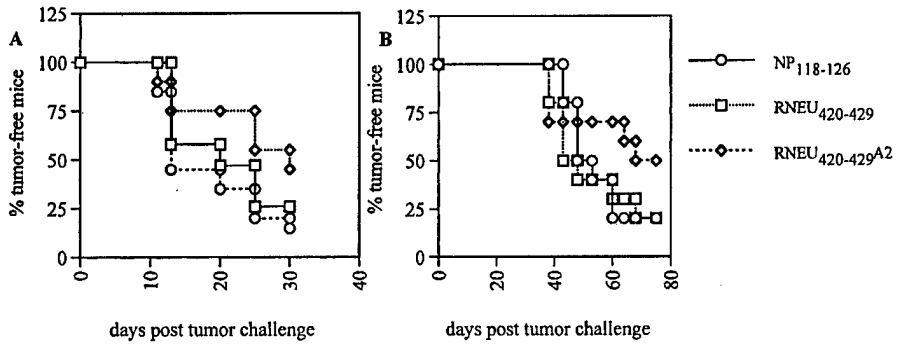

FIGURE 5. Mice immunized with the alanine substituted variant of $RNEU_{420-429}$ show a lag in neu-expressing tumor growth as compared with immunization with wild-type $RNEU_{420-429}$ or the irrelevant peptide. Splenic dendritic cells generated from FVB/N mice were pulsed with 300 μg/ml either $RNEU_{420-429}$, $RNEU_{420-429}A2$, or the lymphocytic choriomeningitis virus $NP_{118-126}$ peptide. Subcutaneous injections (0.1 ml) of 2×10^5 cells were given in each hindlimb on days 0 and 7. Mice were challenged s.c. with NT2 tumor cells on day 14. *A*, FVB/N mice (19–20/group) were given 5×10^6 NT2 cells. *B*, *neu*-N mice (10/group) were given 5×10^4 NT2 cells. Mice were followed twice per week for development of leg tumor.

Identification of a heteroclitic peptide of $RNEU_{420-429}$ that can immunize mice in vivo against outgrowth by the mammary tumor

Identification of the immunodominant neu peptide allowed us to search for altered peptide analogs with potentially enhanced immunogenicity. Altered forms of $RNEU_{420-429}$ were created by substituting alanine at each of the 10 positions. This type of approach has been successful in identifying heteroclitic T cell peptides in both rodent and human settings (6, 49–54). In the majority of cases, substitutions did not enhance recognition. However, when alanine was substituted for glutamate at position 2, this peptide (designated $RNEU_{420-429}A2$) demonstrated markedly improved recognition by the T cell clone FVB/N Vβ4A in a lysis assay as compared with wild-type peptide (Fig. 3). Interestingly, the heteroclitic peptide was found to have a lower binding affinity than the wild-type $RNEU_{420-429}$ peptide in a $T2D^q$ MHC stabilization assay (data not shown), suggesting that its improved stimulatory capacity was instead due to the enhanced stability of the TCR/MHC/peptide complex.

To determine whether this heteroclitic peptide can immunize mice against mammary tumor expressing the natural $RNEU_{420-429}$ epitope, both wild-type $RNEU_{420-429}$ and the heteroclitic variant $RNEU_{420-429}A2$ were used to vaccinate mice. Dendritic cells derived from FVB/N mice were pulsed in vitro with either of these peptides (or with an irrelevant peptide) and then injected s.c. into FVB/N and *neu*-N mice followed by a s.c. NT2 tumor challenge. As shown in Fig. 5, mice immunized with dendritic cells pulsed with the wild-type peptide developed tumor at about the same rate as mice immunized with an irrelevant peptide ($p < 0.15$ for FVB/N mice; $p < 0.39$ for *neu*-N mice). However, FVB/N mice immunized with the heteroclitic peptide showed a lag in tumor growth as compared with FVB/N mice immunized with the irrelevant peptide ($p < 0.012$). Although not statistically significant, *neu*-N mice demonstrated a promising trend toward protection when vaccinated with the heteroclitic peptide ($p < 0.21$).

Discussion

We have identified a MHC-I epitope in rat HER-2/neu, $RNEU_{420-429}$, which is the dominant target of the $CD8^+$ T cell response in FVB/N mice. This peptide, which is contained in the extracellular region of neu, is recognized by all neu-specific $CD8^+$ T cell lines and clones derived from vaccinated FVB/N mice regardless of the neu-targeted vaccine approach used for immunization. This was true even though vaccination induced an oligoclonal neu-specific T cell response as determined by the panel of T cell lines and clones tested expressing several different TCR Vβ types. This was unexpected because the *neu* gene is 4kB in size and encodes for a large protein. However, other studies have reported similar findings demonstrating that a single viral or tumor antigenic epitope is recognized by a panel of T cells derived from immunized mice (8, 55–57). In contrast, neu-specific T cells isolated from patients with neu-expressing breast and ovarian cancer typically recognize multiple epitopes in both the extracellular and intracellular domains of the protein (58–63). Therefore, our data suggest that the FVB/N nontolerized mice recognize neu as a foreign Ag.

FVB/N mice were protected against inoculation with a neu-expressing in vitro tumor line (derived from naturally arising mammary tumors in *neu*-N mice) when T cells specific for this epitope were adoptively transferred. This indicates that $RNEU_{420-429}$ is the immunodominant MHC-I epitope in rat neu that serves as the naturally expressed tumor rejection target on spontaneously arising neu-expressing tumors. It is interesting to note that $RNEU_{420-429}$ differs from the corresponding murine amino acid sequence by 3 aa (64). Furthermore, $RNEU_{420-429}$ specific T cells do not recognize the murine peptide in a ^{51}Cr release lysis assay (data not shown). This probably explains its high degree of immunogenicity in the FVB/N mice.

Vaccination with the $RNEU_{420-429}$ peptide pulsed onto dendritic cells did not demonstrate antitumor immunity. This is not surprising, because vaccinating mice or patients with solely MHC-I tumor epitopes has produced only modest results in most studies. For some Ags, altering the wild-type MHC-I epitope so that it binds more strongly to MHC and/or demonstrates greater recognition in vitro by Ag-specific T cells improves the immunization potential and the clinical outcome (54, 65–67). In this study, we show that vaccinating FVB/N mice with dendritic cells pulsed with a heteroclitic variant of the wild-type epitope also induces improved protection against tumors that express the natural $RNEU_{420-429}$ epitope as compared with immunization with the $RNEU_{420-429}$ epitope itself. *neu*-N mice show a promising trend toward protection when vaccinated with the heteroclitic peptide but not to the degree seen in FVB/N mice. This may reflect the neu-specific tolerance seen in *neu*-N mice. In any case, this regimen proved to be far less efficacious for either strain of mice than our whole-cell vaccine, further supporting the concept that vaccines that induce both CTL and Th cell responses may be more effective than vaccines that only enhance the CTL response (41, 48).

Few studies have been published dissecting the immune responses in *neu*-N mice, which are transgenic for rat *neu* and therefore express a tissue-specific tumor Ag (24, 26–29, 44). We have previously shown that these mice are tolerant to their transgene as compared with the FVB/N strain from which they were derived and that this tolerance is found both in the humoral and T cell arms of the immune response (24). However, we do not yet know the mechanism of this tolerance. It appears from our studies that *neu*-N mice given a whole-cell 3T3neuGM vaccine do not develop T cells specific for the immunodominant peptide in neu, although mice vaccinated with the heteroclitic version of the peptide show a degree of protection from neu-expressing tumor challenge. This may indicate that, although the predominant response to neu in *neu*-N mice is to other, less immunogenic epitopes, T cells specific for the immunodominant peptide may be induced. However, *neu*-N mouse-derived T cells may recognize the immunodominant epitope with a lower avidity as compared with FVB/N-derived T cells. This tolerance may occur in the thymus, in the periphery, or in both compartments. The identification of $RNEU_{420-429}$ as the immunodominant epitope contained in the rat neu protein will facilitate the further characterization of vaccine-induced immune responses in the rat neu-transgenic mouse model. To that end, an MHC/peptide tetramer can be made to identify T cells specific for the immunodominant epitope, because it is known that the restricting MHC molecule is H-$2D^q$. Identification of other epitopes in rat neu is also ongoing.

Acknowledgments

We thank David Woodland for providing TCR Abs, Ted Hansen for providing MHC Abs and L-D^d and L-L^d cells, Susan Ivie for dendritic cell culture, and Eric Lutz for assistance in cloning the H-$2D^q$ cDNA. We also thank Drs. Drew Pardoll and Leisha Emens for their critiques.

References

1. Pan, Z. K., G. Ikonomidis, A. Lazenby, D. Pardoll, and Y. Paterson. 1995. A recombinant *Listeria* monocytogenes vaccine expressing a model tumour antigen protects mice against lethal tumour cell challenge and causes regression of established tumours. *Nat. Med. 1:471.*
2. Pan, Z. K., G. Ikonomidis, D. Pardoll, and Y. Paterson. 1995. Regression of established tumors in mice mediated by the oral administration of a recombinant *Listeria monocytogenes* vaccine. *Cancer Res. 55:4776.*
3. Bright, R. K., M. H. Shearer, and R. C. Kennedy. 1994. Immunization of BALB/c mice with recombinant simian virus 40 large tumor antigen induces antibody-dependent cell-mediated cytotoxicity against simian virus 40-transformed cells: an antibody-based mechanism for tumor immunity. *J. Immunol. 153:2064.*
4. Bright, R. K., B. Beames, M. H. Shearer, and R. C. Kennedy. 1996. Protection against a lethal challenge with SV40-transformed cells by the direct injection of DNA-encoding SV40 large tumor antigen. *Cancer Res. 56:1126.*
5. Schirmbeck, R., W. Bohm, and J. Reimann. 1996. DNA vaccination primes MHC class I-restricted, simian virus 40 large tumor antigen-specific CTL in H-2^d mice that reject syngeneic tumors. *J. Immunol. 157:3550.*
6. Slansky, J. E., F. M. Rattis, L. F. Boyd, T. Fahmy, E. M. Jaffee, J. P. Schneck, D. H. Margulies, and D. M. Pardoll. 2000. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity 13:529.*
7. Tuting, T., J. Steitz, J. Bruck, A. Gambotto, K. Steinbrink, A. B. DeLeo, P. Robbins, J. Knop, and A. H. Enk. 1999. Dendritic cell-based genetic immunization in mice with a recombinant adenovirus encoding murine TRP2 induces effective anti-melanoma immunity. *J. Gene Med. 1:400.*
8. Huang, A. Y., P. H. Gulden, A. S. Woods, M. C. Thomas, C. D. Tong, W. Wang, V. H. Engelhard, G. Pasternack, R. Cotter, D. Hunt, et al. 1996. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. *Proc. Natl. Acad. Sci. USA 93:9730.*
9. Bloom, M. B., D. Perry-Lalley, P. F. Robbins, Y. Li, M. el-Gamil, S. A. Rosenberg, and J. C. Yang. 1997. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med. 185:453.*
10. Bronte, V., E. Apolloni, R. Ronca, P. Zamboni, W. W. Overwijk, D. R. Surman, N. P. Restifo, and P. Zanovello. 2000. Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. *Cancer Res. 60: 253.*
11. Overwijk, W. W., and N. P. Restifo. 2001. Creating therapeutic cancer vaccines: notes from the battlefield. *Trends Immunol. 22:5.*
12. Tuting, T., A. Gambotto, A. DeLeo, M. T. Lotze, P. D. Robbins, and W. J. Storkus. 1999. Induction of tumor antigen-specific immunity using plasmid DNA immunization in mice. *Cancer Gene Ther. 6:73.*
13. Mitchell, M. S., D. Darrah, D. Yeung, S. Halpern, A. Wallace, J. Voland, V. Jones, and J. Kan-Mitchell. 2002. Phase I trial of adoptive immunotherapy with cytolytic T lymphocytes immunized against a tyrosinase epitope. *J. Clin. Oncol. 20:1075.*
14. Rosenberg, S. A., J. R. Yannelli, J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, J. S. Weber, D. R. Parkinson, C. A. Seipp, J. H. Einhorn, and D. E. White. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst. 86:1159.*
15. Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, K. Sakaguchi, E. Appella, J. R. Yannelli, G. J. Adema, T. Miki, and S. A. Rosenberg. 1994. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc. Natl. Acad. Sci. USA 91:6458.*
16. Alexander, M., M. L. Salgaller, E. Celis, A. Sette, W. A. Barnes, S. A. Rosenberg, and M. A. Steller. 1996. Generation of tumor-specific cytolytic T lymphocytes from peripheral blood of cervical cancer patients by in vitro stimulation with a synthetic human papillomavirus type 16 E7 epitope. *Am. J. Obstet. Gynecol. 175:1586.*
17. Steller, M. A., K. J. Gurski, M. Murakami, R. W. Daniel, K. V. Shah, E. Celis, A. Sette, E. L. Trimble, R. C. Park, and F. M. Marincola. 1998. Cell-mediated immunological responses in cervical and vaginal cancer patients immunized with a lipidated epitope of human papillomavirus type 16 E7. *Clin. Cancer Res. 4:2103.*
18. van Driel, W. J., M. E. Ressing, G. G. Kenter, R. M. Brandt, E. J. Krul, A. B. van Rossum, E. Schuuring, R. Offringa, T. Bauknecht, A. Tamm-Hermelink, et al. 1999. Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a phase I-II trial. *Eur. J. Cancer 35:946.*
19. Jager, E., M. Ringhoffer, H. P. Dienes, M. Arand, J. Karbach, D. Jager, C. Ilsemann, M. Hagedorn, F. Oesch, and A. Knuth. 1996. Granulocyte-macrophage-colony-stimulating factor enhances immune responses to melanoma-associated peptides in vivo. *Int. J. Cancer 67:54.*
20. Jager, E., M. Ringhoffer, J. Karbach, M. Arand, F. Oesch, and A. Knuth. 1996. Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and $CD8^+$ cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. *Int. J. Cancer 66:470.*
21. Marchand, M., N. van Baren, P. Weynants, V. Brichard, B. Dreno, M. H. Tessier, E. Rankin, G. Parmiani, F. Arienti, Y. Humblet, et al. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. *Int. J. Cancer 80:219.*
22. Coulie, P. G., V. Karanikas, D. Colau, C. Lurquin, C. Landry, M. Marchand, T. Dorval, V. Brichard, and T. Boon. 2001. A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene *MAGE-3. Proc. Natl. Acad. Sci. USA 98: 10290.*
23. Klausner, R. D. 1999. Studying cancer in the mouse. *Oncogene 18:5249.*
24. Reilly, R. T., M. B. Gottlieb, A. M. Ercolini, J. P. Machiels, C. E. Kane, F. I. Okoye, W. J. Muller, K. H. Dixon, and E. M. Jaffee. 2000. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res. 60: 3569.*
25. Guy, C. T., M. A. Webster, M. Schaller, T. J. Parsons, R. D. Cardiff, and W. J. Muller. 1992. Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA 89:10578.*
26. Cefai, D., B. W. Morrison, A. Sckell, L. Favre, M. Balli, M. Leunig, and C. D. Gimmi. 1999. Targeting HER-2/neu for active-specific immunotherapy in a mouse model of spontaneous breast cancer. *Int. J. Cancer 83:393.*
27. Amici, A., F. M. Venanzi, and A. Concetti. 1998. Genetic immunization against neu/erbB2 transgenic breast cancer. *Cancer Immunol. Immunother. 47:183.*
28. Chen, Y., D. Hu, D. J. Eling, J. Robbins, and T. J. Kipps. 1998. DNA vaccines encoding full-length or truncated Neu induce protective immunity against Neu-expressing mammary tumors. *Cancer Res. 58:1965.*
29. Esserman, L. J., T. Lopez, R. Montes, L. N. Bald, B. M. Fendly, and M. J. Campbell. 1999. Vaccination with the extracellular domain of p185neu prevents mammary tumor development in *neu* transgenic mice. *Cancer Immunol. Immunother. 47:337.*
30. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell 49:273.*
31. Nossal, G. J. 1994. Negative selection of lymphocytes. *Cell 76:229.*
32. Sprent, J., and S. R. Webb. 1995. Intrathymic and extrathymic clonal deletion of T cells. *Curr. Opin. Immunol. 7:196.*
33. Surh, C. D., and J. Sprent. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature 372:100.*
34. Blackman, M., J. Kappler, and P. Marrack. 1990. The role of the T cell receptor in positive and negative selection of developing T cells. *Science 248:1335.*
35. Kisielow, P., H. Bluthmann, U. D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. *Nature 333:742.*
36. Schonrich, G., F. Momburg, M. Malissen, A. M. Schmitt-Verhulst, B. Malissen, G. J. Hammerling, and B. Arnold. 1992. Distinct mechanisms of extrathymic T cell tolerance due to differential expression of self antigen. *Int. Immunol. 4:581.*
37. Ramsdell, F., and B. J. Fowlkes. 1992. Maintenance of in vivo tolerance by persistence of antigen. *Science 257:1130.*
38. Morahan, G., J. Allison, and J. F. Miller. 1989. Tolerance of class I histocompatibility antigens expressed extrathymically. *Nature 339:622.*
39. Godbout, R., B. L. Gallie, and R. A. Phillips. 1984. Characterization of a stable, anchorage-dependent clone obtained from a spontaneously transformed mouse cell line. *In Vitro 20:479.*

40. Bargmann, C. I., M. C. Hung, and R. A. Weinberg. 1986. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. *Cell 45:649.*
41. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. C. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA 90:3539.*
42. Lee, D. R., R. J. Rubocki, W. R. Lie, and T. H. Hansen. 1988. The murine MHC class I genes, *H-2D^q* and *H-2L^q*, are strikingly homologous to each other, *H-2L^d*, and two genes reported to encode tumor-specific antigens. *J. Exp. Med. 168: 1719.*
43. Machiels, J. P., R. T. Reilly, L. A. Emens, A. M. Ercolini, R. Y. Lei, D. Weintraub, F. I. Okoye, and E. M. Jaffee. 2001. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res. 61:3689.*
44. Reilly, R. T., J. P. Machiels, L. A. Emens, A. M. Ercolini, F. I. Okoye, R. Y. Lei, D. Weintraub, and E. M. Jaffee. 2001. The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res. 61:880.*
45. Fearon, E. R., D. M. Pardoll, T. Itaya, P. Golumbek, H. I. Levitsky, J. W. Simons, H. Karasuyama, B. Vogelstein, and P. Frost. 1990. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell 60:397.*
46. Inaba, K., W. J. Swiggard, R. M. Steinman, N. Romani, and G. Sculer. 1998. *Isolation of Dendritic Cells.* Wiley, New York.
47. Deckhut, A. M., W. Allan, A. McMickle, M. Eichelberger, M. A. Blackman, P. C. Doherty, and D. L. Woodland. 1993. Prominent usage of Vβ8.3 T cells in the H-2D^b-restricted response to an influenza A virus nucleoprotein epitope. *J. Immunol. 151:2658.*
48. Greten, T. F., and E. M. Jaffee. 1999. Cancer vaccines. *J. Clin. Oncol. 17:1047.*
49. Herman, J., V. Jongeneel, D. Kuznetsov, and P. G. Coulie. 1999. Differences in the recognition by CTL of peptides presented by the HLA-B*4402 and the HLA-B*4403 molecules which differ by a single amino acid. *Tissue Antigens 53:111.*
50. Sun, D., C. Coleclough, R. Ji, X. Hu, and J. N. Whitaker. 1999. Alanine-substituted peptide ligands differ greatly in their ability to activate autoreactive T-cell subsets specific for the wild-type peptide. *J. Neuroimmunol. 99:105.*
51. Talken, B. L., K. R. Schafermeyer, C. W. Bailey, D. R. Lee, and R. W. Hoffman. 2001. T cell epitope mapping of the Smith antigen reveals that highly conserved Smith antigen motifs are the dominant target of T cell immunity in systemic lupus erythematosus. *J. Immunol. 167:562.*
52. Loftus, D. J., P. Squarcina, M. B. Nielsen, C. Geisler, C. Castelli, N. Odum, E. Appella, G. Parmiani, and L. Rivoltini. 1998. Peptides derived from self-proteins as partial agonists and antagonists of human CD8$^+$ T-cell clones reactive to melanoma/melanocyte epitope MART1$_{27-35}$. *Cancer Res. 58:2433.*
53. Huarte, E., P. Sarobe, J. Lu, N. Casares, J. J. Lasarte, J. Dotor, M. Ruiz, J. Prieto, E. Celis, and F. Borras-Cuesta. 2002. Enhancing immunogenicity of a CTL epitope from carcinoembryonic antigen by selective amino acid replacements. *Clin. Cancer Res. 8:2336.*
54. Rosenberg, S. A., J. C. Yang, D. J. Schwartzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, M. E. Dudley, S. L. Schwarz, P. J. Spiess, et al. 1998. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med. 4:321.*
55. Heukamp, L. C., T. van Hall, F. Ossendorp, J. M. Burchell, C. J. Melief, J. Taylor-Papadimitriou, and R. Offringa. 2002. Effective immunotherapy of cancer in MUC1-transgenic mice using clonal cytotoxic T lymphocytes directed against an immunodominant MUC1 epitope. *J. Immunother. 25:46.*
56. Weidt, G., O. Utermohlen, J. Heukeshoven, F. Lehmann-Grube, and W. Deppert. 1998. Relationship among immunodominance of single CD8$^+$ T cell epitopes, virus load, and kinetics of primary antiviral CTL response. *J. Immunol. 160:2923.*
57. Whitton, J. L., A. Tishon, H. Lewicki, J. Gebhard, T. Cook, M. Salvato, E. Joly, and M. B. Oldstone. 1989. Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. *J. Virol. 63:4303.*
58. Disis, M. L., J. W. Smith, A. E. Murphy, W. Chen, and M. A. Cheever. 1994. In vitro generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu protooncogene protein. *Cancer Res. 54:1071.*
59. Disis, M. L., T. A. Gooley, K. Rinn, D. Davis, M. Piepkorn, M. A. Cheever, K. L. Knutson, and K. Schiffman. 2002. Generation of T-cell immunity to the HER-2/neu protein after active immunization with HER-2/neu peptide-based vaccines. *J. Clin. Oncol. 20:2624.*
60. Fisk, B., B. Chesak, M. S. Pollack, J. T. Wharton, and C. G. Ioannides. 1994. Oligopeptide induction of a cytotoxic T lymphocyte response to HER-2/neu proto-oncogene in vitro. *Cell. Immunol. 157:415.*
61. Ioannides, C. G., B. Fisk, D. Fan, W. E. Biddison, J. T. Wharton, and C. A. O'Brian. 1993. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. *Cell. Immunol. 151: 225.*
62. Knutson, K. L., K. Schiffman, and M. L. Disis. 2001. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *J. Clin. Invest. 107:477.*
63. Rongcun, Y., F. Salazar-Onfray, J. Charo, K. J. Malmberg, K. Evrin, H. Maes, K. Kono, C. Hising, M. Petersson, O. Larsson, et al. 1999. Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. *J. Immunol. 163:1037.*
64. Nagata, Y., R. Furugen, A. Hiasa, H. Ikeda, N. Ohta, K. Furukawa, H. Nakamura, T. Kanematsu, and H. Shiku. 1997. Peptides derived from a wild-type murine proto-oncogene *c-erbB-2/HER2/neu* can induce CTL and tumor suppression in syngeneic hosts. *J. Immunol. 159:1336.*
65. Hoffmann, T. K., D. J. Loftus, K. Nakano, M. J. Maeurer, K. Chikamatsu, E. Appella, T. L. Whiteside, and A. B. DeLeo. 2002. The ability of variant peptides to reverse the nonresponsiveness of T lymphocytes to the wild-type sequence p53$_{264-272}$ epitope. *J. Immunol. 168:1338.*
66. Fong, L., Y. Hou, A. Rivas, C. Benike, A. Yuen, G. A. Fisher, M. M. Davis, and E. G. Engleman. 2001. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proc. Natl. Acad. Sci. USA 98:8809.*
67. Scardino, A., D. A. Gross, P. Alves, J. L. Schultze, S. Graff-Dubois, O. Faure, S. Tourdot, S. Chouaib, L. M. Nadler, F. A. Lemonnier, et al. 2002. HER-2/neu and hTERT cryptic epitopes as novel targets for broad spectrum tumor immunotherapy. *J. Immunol. 168:5900.*
68. Schulz, M., P. Aichele, R. Schneider, T. H. Hansen, R. M. Zinkernagel, and H. Hengartner. 1991. Major histocompatibility complex binding and T cell recognition of a viral nonapeptide containing a minimal tetrapeptide. *Eur. J. Immunol. 21:1181.*